

Basic Mechanisms of Ovarian Function: Germ Cells

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Recent work is reviewed on several molecular aspects of gene expression during oocyte maturation and early development. Several analyses have been carried out on protein synthesis patterns using the high resolution two-dimensional polyacrylamide gel electrophoresis technique. The protein pattern of some 400-500 spots in sea urchin oocytes before and just after fertilization are quite similar. By gastrula, major stage specific protein changes have been noted. In a similar study major protein changes are noted during the meiotic maturation process in mammals. With the use of the single-copy DNA hybridization technique, the quantitative levels of rare mRNA sequence expression have been determined during oogenesis and early development in the sea urchin model. The mature oocyte sequence set of some 37×10^6 nucleotides of information (or potentially 18,500 different proteins) is synthesized during oogenesis and slowly utilized during development. The sea urchin gastrula mRNA is predominantly synthesized by the embryo genome; however, essentially all those gastrula mRNA sequences can also be found in the mature oocyte (maternal) set. It is proposed by Hough-Evans et al. that this maternal sequence set, which is made during oogenesis and both utilized (translated in 10,000-20,000 proteins), and in part, continually synthesized by all stages of the embryo, plays a critical role in the morphogenic formation of a sea urchin embryo.

Introduction

In human beings as well as other mammals, just prior to birth all oogonia in the cortex of the ovary from several million primary oocytes with the 4N amount of DNA, each surrounded by a layer of follicle cells. As the mammalian female matures towards reproductive age, a large number of the immature primary oocytes degenerate. For example, in humans, two million oocytes in the ovary at birth decrease to about 400,000 by adolescence. At reproductive age, when the ovary and primary immature oocyte and follicle cell are exposed to the complex hormonal milieu necessary for oocyte maturation, the differentiation and growth of maturing oocytes require a high level of biosynthetic activity. Presumably this increase in gene activity is due either to endogenous information expressed within the oocyte or to information that is transferred from the follicle cells to the maturing oocyte, potentially altering the oocyte's synthetic capacity.

One approach to understanding the mechanisms

involved in the maturation of oocytes in mammals would be to define some of the gene products produced during this active synthetic period (1), to ask what kinds of and what numbers of genes are expressed at both the transcription and translation levels, and to ask how that information is utilized during early development. During this process, one could analyze the pattern of protein synthesis and modification at various stages, assess the number of different structural genes (messenger RNA) transcribed, and analyze how these genes participate in and overlap with the pattern of embryo gene expression by using the techniques of nucleic acid hybridization (2).

Protein Synthesis Patterns During Oocyte Maturation and After Fertilization: Analysis by Two-Dimensional Polyacrylamide Gel Electrophoresis (IEF-SDS Gels)

With the availability of high specific activity amino acid precursors such as ^{35}S -methionine ($> 200 \text{ Ci/mmol}$) and high resolution separation methods, involving isoelectric focusing in one di-

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mension and molecular weight or size separation in the other, a general pattern for proteins synthesized at one stage of oocyte maturation can be compared with the patterns at earlier or later stages. A recent article by Brandhorst (3) indicated at least 400 resolvable newly synthesized proteins (from the soluble or 105,000g supernatant) in the mature sea urchin oocyte *Lytechinus pictus*. Upon fertilization, he found that the rate of total protein synthesis increased greatly; however, when the soluble proteins were analyzed on two-dimensional gels, only a few new spots out of the 400 or so were unique to the fertilized state. Therefore, for these soluble proteins the activation of the stored maternal messenger RNA appears to be mainly a quantitative rather than a qualitative change. However, by gastrulation major differences appear in the protein profiles as analyzed by this two-dimensional technique (3-5).

The ^{35}S -methionine label/two-dimensional gel electrophoresis technique indicates both major qualitative and quantitative changes in protein synthesis profiles during the meiotic maturation of mammalian oocytes *in vitro* (6). In these experiments total labeled proteins were analyzed rather than just the soluble fraction. Most of the protein changes occur before the germinal vesicle breaks down. Apparently the nucleoplasm is necessary for "activating" or "triggering" the observed changes in the pattern of protein synthesis during meiotic maturation in mammalian oocytes. It was also noted by Schultz and Wassarman (6) that germinal vesicle breakdown and the subsequent change in protein synthesis could be blocked by dibutyl 3',5'-cyclic AMP. Thus, unlike lower animals such as amphibians, in which it appears as if meiotic maturation can occur without nucleoplasm (7), mammalian oocytes require "pre-germinal vesicle breakdown" nuclear information for meiotic oocyte maturation (6).

Since it is difficult to obtain sufficient biological material from mammalian sources for molecular experiments, much of our knowledge about gene expression during oogenesis and the early stages of embryological development has come from the sea

urchin model. Large quantities of mature oocytes can be collected by injecting 0.5M KCl into the gravid female. Other stages of oogenesis can be obtained by dissection and are relatively homogeneous due to the seasonal nature of the egg maturation process in sea urchins.

Use of the Single Copy DNA-RNA Hybridization to Quantitate Levels of Structural Gene Expression

Isolation of single-copy or unique DNA has previously been described (2). Basically, whole sheared DNA [400 nucleotide (NT)] is incubated at a given salt (0.18M Na⁺) and temperature (60-70°C) for increasing times and at different concentrations [C_0t = moles of nucleotides per liter \times time (sec)] until the repetitive sequences in the genome have reannealed. The duplexes can be physically separated from the single-copy DNA or unannealed DNA by hydroxyapatite chromatography. One can now label the single-copy DNA by using a "nick-repair" system and *E. coli* DNA polymerase I (8).

With the labeled single-copy DNA one may hybridize various RNA preparations to the radioactive DNA and then determine from the level of hybridization the expressed genomic output in that RNA sample. By its nature, the technique only looks at the rare, scarce, or low abundant RNA sequences in the RNA preparation under investigation. For example, Galau et al. (8) isolated polysomal messenger RNA from sea urchin gastrula and hybridized this messenger RNA preparation to radioactive single-copy DNA. They found that 0.67% of the ^3H -DNA could form hybrids. Assuming only 50% of the DNA is transcribed in eukaryotic cells, 1.34% (0.67/.5) of the total genetic information of the sea urchin genome (17×10^6 NT or equivalent to 8,500 different mRNA sequences of an average of 2000 NT in length) is found in the gastrula mRNA sequence set.

Table 1. Hybridization of ^3H -mDNA_{GAS} and ^3H -null DNA_{GAS} with gastrula, adult intestine, and tubefeet messenger RNA.^a

Tissue mRNA	^3H -mDNA _{GAS} in hybrid (terminal value), %	Complexity (10 ⁶ NT)	^3H -null DNA _{GAS} , %	Complexity (10 ⁶ NT)	Total complexity (10 ⁶ NT)
Gastrula	57	17	0	0	17
Intestine	7.2	2.1	0.26	3.7	5.8
Tubefeet	9.3	2.7	≤ 0.03	0-0.4	2.5-3.3

^a Adapted from Galau et al. (9).

Isolation of a Single-Copy ^3H -DNA Probe to Analyze Expressed Structural Gene Overlap during Later Stages of Development in the Sea Urchins

Recently, Galau et al. (9) took whole ^3H -labeled single-copy DNA and hybridized it to gastrula messenger RNA. They then separated the hybridized DNA from the unhybridized DNA using hydroxyapatite chromatography and then repurified the two DNA samples. Interesting questions may now be asked about structural gene overlap and stage specific structural gene expression by hybridizing messenger RNA (mRNA) from various other developmental stages to the gastrula specific DNA (mDNA_{GAS} and null DNA_{GAS} (i.e. DNA that has no gastrula mRNA sequences) and found 2×10^6 NT of complexity (equivalent to about 1000 structural proteins) in the mDNA_{GAS} and about 3.7×10^6 NT of complexity in the null DNA_{GAS}.

Similarly, if mRNA from sea urchin tube feet is hybridized to the two DNA samples, one finds about 2.7×10^6 NT of information in the tube feet mRNA hybridizing to mDNA_{GAS} but very little is observed in the null DNA_{GAS} ($0-0.4 \times 10^6$ NT). The basic conclusion from these initial experiments is that approximately 1000–2000 structural genes (make a protein product) overlap from some adult tissues and the gastrula stage sea urchin structural gene set. These sequences have been referred to as the “house keeping structural genes” (9). These results are summarized in Table 1.

Hybridization of Oocyte RNA and Ovary mRNA to mDNA_{GAS} and Null DNA_{GAS}

What is of main interest now is what overlap in structural gene expression exists between the RNA sequences being transcribed and translated in the gastrula and the maternal RNA sequences made during oocyte maturation. Galau et al. (9) found that

RNA from oocytes (total RNA, since there are few polysomes) hybridized to mDNA_{GAS} to the same extent as gastrula mRNA or to the equivalent of 17×10^6 NT of complexity. This suggests that most all the sequences for the scarce mRNA class being synthesized in the gastrula embryo were also made during oocyte maturation. When the oocyte RNA was hybridized to the null DNA_{GAS}, 20×10^6 NT of expressed genetic information or the equivalent of 10,000 structural genes not found in the gastrula mRNA population but made sometime during oogenesis was determined from the hybridization saturation value. This suggests that the single cell maturing oocyte gene expression pattern is over twice as complex in its gene expression of the high complexity sequence set as the differentiating 600-cell gastrula sea urchin embryo.

One may ask, however, how do we know all this genetic information in the mature oocyte is ever translated into proteins since the RNA hybridized to the mDNA_{GAS} and null DNA_{GAS} was total RNA and not polysomal? The following suggests that much of the RNA sequence information in the mature oocyte was earlier associated in the ovary with polysomes. Polysomal RNA was isolated from ovaries in which the mature oocytes were removed and a significant but unknown fraction of immature oocytes remained in the whole ovary. This polysomal RNA preparation was hybridized to mDNA_{GAS} and found to overlap 80% with gastrula polysomal RNA (a genetic complexity of 13×10^6 NT or equivalent to 6500 structural proteins synthesized in the ovary and in gastrula embryo). By subtraction then (17×10^6 NT for gastrula minus 13×10^6 NT overlap in the ovary), 4×10^6 NT or 2000 structural genes are found in the gastrula and not in the ovary that contains mostly immature oocytes. When ovary polysomal RNA is hybridized to null DNA_{GAS}, hybridization saturation values indicate a complexity of 6.7×10^6 NT. The total complexity of ovary polysomal RNA is then about 20×10^6 NT ($6.7 + 13$). A unique sequence set and equivalent to 3350 structural genes (6.7×10^6 NT/2000 NT in length) is thus found in the ovary and not in the gastrula mRNA sequence set. These results are summarized in Table 2.

Table 2. Hybridization of ^3H -mDNA_{GAS} and ^3H -null DNA_{GAS} with mature oocyte RNA and ovary mRNA.^a

Tissue RNA	^3H -mDNA _{GAS} in hybrid (terminal value), %	Complexity (10^6 NT)	No. of structural genes	^3H -null DNA _{GAS} in hybrid, %	Complexity (10^6 NT)	No. of structural genes	Total complexity (10^6 NT)
Gastrula mRNA	57	17	8,500	0	0	0	17
Mature oocyte RNA	57	17	8,500	1.42	20	10,000	37
Ovary	45	13	6,500	0.47	6.7	3,350	20

^a Adapted from Galau et al. (9).

Is the Complex Structural Sequence Set (8500 Different Proteins) Found in Gastrula Maternal or Embryonic in Origin?

One may ask if the rare class sequence set found on embryo (blastula/gastrula) polysomes is maternal in origin (from the mature oocyte RNA set) or in fact newly synthesized by the embryo. By labeling gastrula *in vivo* with ^3H -uridine, many experiments have indicated, that most of the polysomal RNA is synthesized by the embryo genome. However, the labeling experiments apply only to the prevalent or abundant mRNA sequences. Galau et al. have recently reinvestigated this question of embryonic or maternal origin of both the prevalent, as well as the rare mRNA sequences during embryonic development (10). By labeling embryos (blastula and gastrula) with ^3H -guanosine and measuring and correcting for ^3H -GTP pools, they found that the half-life of embryo-synthesized messenger RNA is about 6 hr. It takes approximately 30 hr at 15°C to reach early gastrula.

By hybridizing the ^3H -labeled RNA to single-copy DNA, they found that the specific activity of the RNA in the hybrids was about the same as that of the total labeled ^3H -RNA. The important conclusion from these experiments is that the rare or high complexity (i.e., thousands of different structural genes) messenger RNA is synthesized from the embryo genome and not maternal in origin. Thus, the large overlap or sequence homology between embryo and oocyte mRNA discussed earlier indicates that many of the same structural genes active in protein synthesis during oogenesis are also actively transcribed and translated in the gastrula embryo.

Hybridization of ^3H -Mature Oocyte DNA (^3H -DNA_{MO}) with Various RNA Preparation from Immature Oocytes and Later Embryonic Stages in the Sea Urchin

In a similar manner, an analysis of the complex RNA class made during oogenesis (mature oocyte RNA set) and its utilization during embryonic development can be made by hybridizing mature oocyte RNA to total ^3H -single-copy DNA, isolating the RNA-DNA hybrid material by hydroxyapatite, and purifying the specific DNA probe (11). The ^3H -mature oocyte DNA probe (^3H -DNA_{MO}) can now be used in the following experiment. Hough-Evans et al. (11) took mature oocyte RNA and found it

hybridized to about 3% of the single-copy DNA or equivalent to 37×10^6 NT of information. This value is consistent with the hybridization of mature oocyte RNA with ^3H -mDNA_{GAS} (17×10^6 NT) and ^3H -null DNA_{GAS} (20×10^6 NT) discussed earlier ($20 + 17 = 37$).

The first question that can be asked with the mature oocyte ^3H -DNA probe is, when during oogenesis is this complex set of sequences synthesized? Hough-Evans et al. (11) isolated RNA from ovaries which contain > 90% immature oocytes (previtellogenic and early vitellogenic) and found it to contain 70% of the mature maternal sequence set [$(0.7)(37 \times 10^6) = 26 \times 10^6$ NT]. Therefore a large part of the maternal RNA sequence set is being synthesized at this early stage of oocyte maturation.

Over the next few months in which the oocyte continues development towards maturation, some 30% of the mature oocyte sequence set (11×10^6 NT of information, i.e., $0.3 \times 37 \times 10^6$) must be expressed by the single (4N) oocyte genome. The large diversity of sequence information expressed during oocyte maturation may take such a long time because of the large demand on the single (4N) genome of the maturing oocyte.

By hybridizing various RNA preparations from whole cytoplasm or from isolated polysomes of sea urchin embryos at different stages of development to the ^3H -DNA_{MO} probe, an assessment may be made of the persistence of the maternal RNA sequences during these early stages. For example, total cytoplasmic RNA from 16-cell cleavage stage embryos hybridizes to the same extent as mature oocyte RNA and therefore the early cleavage stage still contains all 37×10^6 NT of information. However, if polysomal or mRNA is used from the 16-cell stage, only 73% of the mature oocyte RNA hybridization value was reached with the ^3H -DNA_{MO} probe or 27×10^6 NT. Hough-Evans et al. (11) have then done similar experiments with blastula cytoplasmic and polysomal RNA and found about 85% and 49% and of the mature oocyte hybridization values for cytoplasmic and polysomal RNA, respectively. By the pluteus stage, the cytoplasmic and polysomal RNA values for hybridization to the ^3H -DNA_{MO} probe are about 40% of the mature oocyte RNA value. The cytoplasmic and polysomal RNA values are more similar at this later stage. The discrepancy between cytoplasmic and polysomal values at the earlier stages suggest that the maternal information is present (100% in 16-cell, 85% in blastula) in the cytoplasm but is being "selected" against at the translation machinery since by the blastula only about 50% of the mature oocyte sequences can be found on polysomes. There is still a

Table 3. Hybridization of ^3H -DNA_{MO} (mature oocyte specific sequences) with immature oocyte total RNA and with various embryonic stage RNAs isolated from cytoplasm and polysomes.^a

Tissue	RNA source	Complexity (10 ⁶ NT)	No. of structural genes (2000 NT ea.)	Mature oocyte (MO), %
Mature oocytes	Total	37	18,500	100
Immature oocytes	Total	26	13,000	72
16-cell embryo	Cytoplasmic mRNA	37	18,500	100
		27	13,500	73
Blastula	Cytoplasmic mRNA	31	15,500	85
		18	9,000	49
Pluteus	Cytoplasmic mRNA	16	8,000	44
		13-15	7,000	38-42

^a Adapted from Hough-Evans et al. (11).

large pool of maternal sequence information in the cytoplasm that may be translated at a slower rate than the information synthesized by the embryo genome and associated with polysomes. These results are summarized in Table 3 and indicate the importance of post-transcriptional as well as post-translational utilization of the maternal sequence information made by both the oocyte and the embryo.

Significance of Rare mRNA Sequences during Oogenesis and Early Development

The rare RNA class represents about 10% of the total mRNA at most stages and has enough genetic information to synthesize 10,000–20,000 different protein species. As stated earlier, when protein synthesis is analyzed by two-dimensional electrophoresis, about 400 spots are detected. These obviously represent the translation (and modification) of the prevalent class of messenger RNA's. Therefore, what is the role or biological significance of the rare mRNA species? It has been calculated that the rare mRNA's are present in about 1 to 30 copies per cell. Galau et al. (12) have considered the significance of the rare mRNA sequences in rat liver as a model since a large number of proteins have been analyzed in this organ. Their basic conclusion based on some theoretical calculations is that there are many proteins (enzymes, etc.) in the liver that would require a steady-state concentration of cell specific mRNA of from 1 to 30 copies per cell and would therefore belong to the rare mRNA class. Also one should consider the large class of nuclear heterogenous nonhistone protein mRNAs as well as other potential regulatory protein mRNAs which may be needed during development as potential members of the rare mRNA class.

Another viewpoint on the significance and utilization of the rare mRNA in oogenesis and early development is that during oogenesis and early development there is the translation and slow accumulation of a large set of rare proteins (10,000–20,000) that are required for morphogenesis (11). These "morphogenesis proteins" begin their accumulation early in oogenesis, long before they are utilized in building a sea urchin embryo. The utilization of the "morphogenesis proteins" would be a slow sequential process requiring relatively few embryo specific structural gene products.

Whatever the role of the rare mRNA class in oogenesis and development, their high content of expressed genetic information offers a challenge to understanding how oogenesis lays the foundation for embryonic development.

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